ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Oct. 2006, p. 3470–3472 Vol. 50, No. 10
0066-4804/06/$08.00+0 doi:10.1128/AAC.00577-06

# Maribavir Antagonizes the Antiviral Action of Ganciclovir on Human Cytomegalovirus

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Received 10 May 2006/Returned for modification 19 June 2006/Accepted 25 July 2006

**The cytomegalovirus (CMV) UL97 kinase inhibitor maribavir antagonized the anti-CMV effect of ganciclovir, increasing the ganciclovir 50% inhibitory concentration against a sensitive strain by up to 13-fold. Antiviral activities of foscarnet and cidofovir were unaffected by maribavir.**

Maribavir (MBV; 1263W94) is a human cytomegalovirus (CMV) UL97 kinase inhibitor (1) undergoing clinical trials. The antiviral action of ganciclovir (GCV), the most widely used current anti-CMV drug, depends on its initial phosphorylation by the viral UL97 kinase. The UL97-inhibitory effect of MBV is predicted to impair the phosphorylation of GCV, thereby antagonizing its antiviral effect. Yet the antiviral activities of MBV and GCV in combination were described as additive or indeterminate (8, 11). Here, we tested the effect of MBV on GCV activity against sensitive and resistant CMV strains. As controls, we studied the effect of MBV on foscarnet (FOS) and cidofovir (CDV), drugs not involving UL97-mediated phosphorylation.

CMV strain AD169 was used to derive a drug-sensitive strain (T2233) containing a secreted alkaline phosphatase (SEAP) reporter gene for rapid viral quantitation (4). GCV-resistant SEAP-expressing strains T2258 and T2260 containing UL97 mutations C592G and L595S, respectively, and an MBV-resistant SEAP-expressing strain (T2264) containing UL97 mutation L397R have also been described previously (4, 5). GCV (Roche), FOS (Astra), and CDV (Gilead) were obtained from their respective manufacturers. MBV was obtained from Glaxo-SmithKline. CMV was cultured in locally derived human embryonic lung (HEL) fibroblasts (passages 10 to 20) or human foreskin fibroblasts (HFF; passages 20 to 30) as previously described and compared with commercial cell cultures (5). SEAP yield reduction assays were performed as recently described (4, 5). Briefly, 6 wells of a 24-well culture of fibroblasts were inoculated with a cell-free virus stock at a multiplicity of infection of 0.01 to 0.03. One well was a no-drug control, and the rest were cultured with twofold serial dilutions of the drug to be tested. In some experiments, a fixed concentration of a second drug was added to all six wells. Five to 6 days after inoculation, aliquots of culture supernatant were assayed for SEAP activity. The drug concentration required to reduce the SEAP activity to 50% of the no-drug control value ($EC_{50}$) was calculated by fitting an exponential curve to the SEAP activities measured in the drug-containing wells.

The SEAP yield reduction $EC_{50}$ of each of the drugs (MBV, GCV, FOS, and CDV) acting alone against strain T2233 is shown in Table 1 and is consistent with previously published data (4, 5), with the $EC_{50}$ of MBV much higher in HFF than in HEL cells. Strains T2258 and T2260 showed a level of GCV resistance in HEL cells similar to previous findings obtained with HFF (4, 6). Strain T2264 shows >100-fold increased MBV resistance over the baseline strain T2233 MBV resistance, consistent with previous findings (1, 5).

Checkerboard assays of MBV paired with GCV, FOS, and CDV were performed as a six-by-six or six-by-eight matrix with 24-well HFF cultures inoculated with CMV strain T2233 at a multiplicity of infection of 0.01 to 0.02. As additional controls, checkerboard assays were also done with HFF and GCV-FOS, GCV-CDV, and FOS-CDV. Virus was cultured with drug combinations (e.g., MBV and GCV) in increasing twofold concentrations on each axis of the matrix, and culture supernatants were assayed for SEAP activity after 5 to 6 days. The first row and column of the matrix contained only one of the drugs and were used to determine the $EC_{50}$ of each drug alone. Assays were set up in duplicate (quadruplicate in the case of MBV-GCV), and the mean SEAP values for each drug combination were used to calculate the $EC_{50}$s of drug A in the presence of various amounts of drug B and vice versa. The fractional inhibitory concentration (FIC), defined as the $EC_{50}$ of drug A in the presence of drug B divided by the $EC_{50}$ of drug A alone, was calculated for each drug of the interacting pair. The sum of the FICs for drug A and drug B (ΣFIC) was used to determine the drug interaction (7). Synergism was defined as a ΣFIC of <0.5, and antagonism was defined as a ΣFIC of >4, with all values in between as indeterminate, according to guidelines established for this and other journals (10).

Checkerboard assay results showed the following features. With each combination of the current drugs GCV, FOS, and CDV, the $EC_{50}$ of drug A in the presence of drug B (at a concentration near the $EC_{50}$ of drug B alone) differed less than twofold from the $EC_{50}$ of drug A by itself. FICs ranged from 1.05 to 1.64, and ΣFICs ranged from 2.3 to 3.0, which are indeterminate for synergy or antagonism. With the MBV drug combinations, the $EC_{50}$s of FOS and CDV were almost unchanged in the presence of 6, 12, or 24 μM MBV (FIC range, 0.9 to 1.1). However, the $EC_{50}$ of GCV was increased up to 14-fold with the addition of MBV. At therapeutic GCV concentrations of 2 to 16 μM, the viral SEAP yield increased by up to twofold when 8 μM MBV was added, and an approximately fourfold increase in the GCV concentration was required to

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TABLE 1. Effect of maribavir on $EC_{50}$s of other anti-CMV drugs

| Cell type and strain | UL97 genotype | Drug A | Drug B (concn [μM]) | $EC_{50}$ (μM) of drug A (no. of replicates)[a] | FIC | MBV ΣFIC[b] |
|---|---|---|---|---|---|---|
| HFF | | | | | | |
| T2233 | WT[c] | GCV | None | 1.0 ± 0.4 (28) | | |
| | | GCV | MBV (0.04) | 1.6 ± .76 (13) | 1.5 | >3.9 |
| | | GCV | MBV (0.16) | 4.5 ± 1.2 (19) | 4.3 | **>6.7** |
| | | GCV | MBV (0.64) | 6.6 ± 3.3 (17) | 6.3 | **>8.7** |
| | | GCV | MBV (2.5) | 8.3 ± 2.3 (12) | 8.0 | **>10** |
| | | GCV | MBV (5) | 14 ± 4.5 (15) | 13 | **>15** |
| | | GCV | MBV (10) | 13 ± 3.0 (7) | 13 | **>15** |
| | | FOS | None | 45 ± 8.3 (15) | | |
| | | FOS | MBV (10) | 39 ± 7 (8) | 0.9 | 0.9 |
| | | CDV | None | 0.26 ± 0.07 (14) | | |
| | | CDV | MBV (10) | 0.27 ± .18 (7) | 1.0 | 1.1 |
| | | MBV | None | 13 ± 3.6 (16) | | |
| | | MBV | GCV (1) | >32 (6) | >2.4 | |
| | | MBV | FOS (40) | 0.47 ± 0.3 (16) | 0.04 | |
| | | MBV | CDV (0.3) | 0.41 ± 0.05 (5) | 0.03 | |
| HEL cells | | | | | | |
| T2233 | WT | GCV | None | 0.55 ± 0.18 (12) | | |
| | | GCV | MBV (0.04) | 1.2 ± 0.19 (6) | 2.2 | **4.1** |
| | | GCV | MBV (0.16) | 2.6 ± 0.89 (13) | 4.7 | **6.6** |
| | | GCV | MBV (0.32) | 4.9 ± 1.0 (11) | 8.9 | **11** |
| | | FOS | None | 39 ± 10 (7) | | |
| | | FOS | MBV (0.16) | 27 ± 9 (7) | 0.7 | 1.6 |
| | | CDV | None | 0.36 ± 0.04 (6) | | |
| | | CDV | MBV (0.16) | 0.29 ± 0.12 (6) | 0.8 | 1.6 |
| | | MBV | None | 0.10 ± 0.03 (51) | | |
| | | MBV | GCV (0.5) | 0.19 ± 0.01 (4) | 1.9 | |
| | | MBV | FOS (40) | 0.09 ± 0.03 (7) | 0.9 | |
| | | MBV | CDV (0.4) | 0.08 ± 0.00 (4) | 0.8 | |
| T2264 | L397R ($MBV^r$) | MBV | None | 24 ± 9.1 (7) | | |
| | | GCV | None | 1.9 ± 0.73 (6) | | |
| | | GCV | MBV (10) | 1.9 ± 0.74 (4) | 1.0 | |
| | | FOS | None | 31 ± 14 (7) | | |
| | | FOS | MBV (10) | 48 ± 13 (4) | 1.6 | |
| | | CDV | None | 0.47 ± 0.09 (4) | | |
| | | CDV | MBV (10) | 0.45 ± 0.3 (6) | 1.0 | |
| T2258 | C592G (low-grade $GCV^r$) | MBV | None | 0.2 ± 0.04 (5) | | |
| | | GCV | None | 2.5 ± 1.4 (5) | | |
| | | GCV | MBV (0.2) | 7.2 ± 1.1 (4) | 2.9 | |
| T2260 | L595S ($GCV^r$) | MBV | None | 0.25 ± 0.08 (5) | | |
| | | GCV | None | 7.9 ± 3.2 (5) | | |
| | | GCV | MBV (0.2) | 8.6 ± 5.4 (7) | 1.1 | |

[a] $EC_{50}$s are for drug A in the presence of drug B and are shown as the mean ± the standard deviation.
[b] Values of >4, defining drug antagonisms, are in bold.
[c] The phenotype associated with the genotype is in parentheses.

achieve a similar antiviral effect. For example, the measured SEAP activity (relative light units) was 13,786 ± 1,928 (mean ± standard deviation) with 8 μM GCV by itself, 26,982 ± 884 with 8 μM GCV and 8 μM MBV, and 14,362 ± 963 with 32 μM GCV and 8 μM MBV. These results fulfill the basic definition of antagonism as a reduced effect of a combination of drugs in comparison with the effect of the most effective individual substance (7). With no other drug combination did the addition of any amount of a second drug to the first drug cause an increase in the viral SEAP yield.

In addition to the HFF checkerboard assays, multiple additional determinations of ΣFIC were performed for specific MBV-drug combinations in both HEL cell and HFF cultures. Results are shown in Table 1. FICs and ΣFICs derived from assays of HFF (Table 1) were consistent with the checkerboard data. GCV $EC_{50}$s increased as MBV concentrations were escalated, ultimately reaching a FIC of 13 and ΣFICs of >15, which easily qualify for antagonism (7, 10). Comparable ΣFICs for MBV-FOS and MBV-CDV were close to 1, indeterminate for synergy or antagonism. In HEL cells (Table 1), where the MBV $EC_{50}$ is much lower, the GCV $EC_{50}$ was increased ninefold in the presence of 0.32 μM MBV. Combined with the FIC of MBV at a GCV concentration of 0.5 μM, the ΣFIC is 11, which again exceeds the criterion for antagonism. In HEL cells, concentrations of MBV beyond 0.32 μM reduced the viral yield by >75% (5), making it difficult to assess the incre-

mental effect of a second drug. Comparable ΣFICs for MBV-FOS and MBV-CDV (both at 1.6) showed no evidence of synergy or antagonism.

For strain T2260, containing the UL97 mutation L595S, which decreases GCV phosphorylation by >80% (2), there was a 14-fold increase in the GCV $EC_{50}$ over that for a T2233 control, a value that was not significantly affected by the presence of MBV (FIC = 1.1; Table 1). For strain T2258, containing UL97 mutation C592G, which confers low-grade GCV resistance (6), there was a 2.9-fold increase in the GCV $EC_{50}$ in the presence of 0.2 μM MBV (Table 1). The sensitivities of MBV-resistant strain T2264 to GCV, CDV, and FOS were unaffected by the presence of 10 μM MBV.

MBV is expected to antagonize the antiviral action of GCV by preventing its initial phosphorylation by the UL97 kinase. Published data obtained with the purified UL97 enzyme show that MBV at 1 μM inhibits UL97 autophosphorylation by >99% (1) but offer no direct evidence that MBV prevents GCV phosphorylation in infected cells. However, UL97 mutants lacking autophosphorylation capability are also defective for GCV phosphorylation (9). Evidence of MBV antagonism of GCV is more apparent in HFF cells, where concentrations of MBV that are below its $EC_{50}$ achieve near-maximal antagonism of GCV activity (Table 1). This is consistent with a model in which HFF cellular kinases are substituting for the normal biological function of UL97 but not its ability to phosphorylate GCV.

Methodological and interpretive differences likely explain the discordance of our findings with the published literature regarding the interaction between GCV and MBV. The ΣFIC method used here is relatively insensitive given the wide range of indeterminate ΣFICs (10), but the findings obtained with GCV-MBV were unambiguous. One report (11) concluded that GCV and MBV had an additive interaction. Comparisons were made with a GCV-GCV checkerboard, whereas we used other drug combinations as controls. Another study (8) using a different mathematical model found an indeterminate interaction between GCV and MBV while describing strong synergy between the drug pairs MBV-FOS and MBV-CDV, which is inconsistent with findings obtained in this study on the basis of ΣFIC calculations or with results from the other study (11). Also, the two previous studies (8, 11) used MRC-5 HEL cells for their combination antiviral assays. As shown in Table 1, CMV is more sensitive to MBV in HEL cells, thus making it more difficult to measure the antiviral effect of an added second drug.

From the results presented here, it seems that combination therapy with MBV and GCV for sensitive strains has no advantage over using GCV alone and could be counterproductive. The interplay of kinase activities gives some interesting results with UL97 mutants that are resistant to either of these drugs. Strains containing UL97 mutations conferring GCV resistance are already deficient in GCV phosphorylation (probably because of altered GCV substrate specificity), thus making it less important that MBV also reduces GCV phosphorylation. The UL97 L397R mutant retains its usual GCV sensitivity in the presence of MBV because its UL97 activity is resistant to inhibition by MBV, so that phosphorylation of GCV can occur. Thus, one component of GCV-MBV combination therapy should theoretically retain antiviral activity against either type of resistant UL97 mutant. However, if MBV resistance results from genetic changes other than in UL97, such as in UL27 (3), a GCV-MBV combination would be predicted to be worse than GCV alone.

This work was supported by NIH grant AI-39938 and by Department of Veterans Affairs research funds.

We thank Laura Van Wechel for technical assistance with the SEAP assays.

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